−原著−

口腔粘膜上皮細胞培養上清で培養した口腔粘膜線維芽細胞の形質に関する検討

マザビンビンタモニル

新潟大学大学院医歯学総合研究科　口腔解剖学分野

Characterization of an oral fibroblast phenotype cultured in oral keratinocyte-conditioned medium

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Key words: Oral fibroblast, oral keratinocyte, conditioned medium, phenotype, monolayer culture

Abstract:

Fibroblasts in monoculture grown in serum-containing medium are highly-proliferating. However, data suggested that use of static fibroblasts provides a better model to study biological phenomena than proliferating fibroblasts. Since previous studies have stated keratinocyte-conditioned medium reduced fibroblast proliferation, this study aimed to examine if the oral keratinocyte (OK) -conditioned medium (CM) can decrease oral fibroblasts (OF) proliferation and to characterize their phenotype. Primary human OK and OF were grown in a completed EpiLife® (0.06mM Ca^{++}) and Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum (DMEM-CS), respectively. OK-CM was conditioned for 24 hours in a near-confluent OK culture. OFs plated in a micro-plate well were cultured with DMEM-CS, serum-free DMEM (SF-DMEM) and OK-CM for up to 96 hours. Proliferation rate and cell cycle profile were analyzed using a MTT assay and a fluorescence-activated cell sorter. The "phenotypic changes" of OFs were determined by the activity of senescent-associated β-galactosidase (β-gal) and the secreted protein levels including keratinocyte growth factor (KGF), human type I collagen and matrix metalloproteinase-1 (MMP-1) measured by enzyme-linked immunosorbent assay (ELISA). The proliferating rate and the proportion of cells in S phase were significantly lower when cells were cultured in OK-CM. The β-gal activity suggested OFs in OK-CM still had proliferating potential. ELISA assay showed OFs cultured in OK-CM produce KGF and MMP-1 as did OFs grown in DMEM-CS while their ability to produce type I collagen was significantly lower than OFs in DMEM-CS. This study suggested the OK-CM generated a quiescent OF population possessing the characteristic of extracellular matrix degradation rather than synthesis.

MTT : 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

日本語要旨：

生体反応の研究に用いる線維芽細胞は増殖している細胞ではなく休止期のものが好ましい。上皮細胞の培養上清に線維芽細胞の増殖抑制効果があることが報告されている。本研究の目的は口腔粘膜上皮細胞の培養上清が培養口腔粘膜線維芽細胞の増殖性を抑え，細胞形質を変化させるかを検証することである。ヒト初代口腔粘膜上皮細胞（OK）と線維芽細胞（OF）はそれぞれ EpiLife と 10％ウシ胎児血清含有ダルベッコ改変イーグル培地（DMEM）（DMEM-CS）中で培養した。コンフルエントに近い口腔粘膜上皮細胞を 24 時間培養したものを上清とした。口腔粘膜線維芽細胞は DMEM-CS，DMEM 培地単独（SF-DMEM），口腔粘膜上皮細胞培養上清（OK-CM）の 3 種類の培地で，最長 96

時間培養した。細胞増殖能と細胞周期はそれぞれ MTT assay とセルソーターで解析した。細胞形質変化の分析はさらに，βガラクトシダーゼ活性発現とエライザ法（ELISA）による各種タンパク質（ケラチノサイト増殖因子（KGF），タイプⅠコラーゲン，マトリックスメタロプロテアーゼ-1（MMP-1））分泌量で検討した。細胞増殖能とS期の細胞の割合は有意に OK-CM で培養した細胞で低下しており，G0/G1 期停止による細胞増殖抑制の可能性が示唆された。また OK-CM 培養では，βガラクトシダーゼ活性を示した細胞は少数で，口腔粘膜線維芽細胞は休止期にいるが，増殖能が失われていないことを示した。KGF と MMP-1 の産生量は，DMEM-CS で培養した細胞と同等であったのに対し，Ⅰ型コラーゲンの産生量が有意に低かった。本研究から，OK-CM は培養口腔粘膜線維芽細胞の増殖能を抑え，かつ細胞外基質分解性の細胞形質となることが示唆された。

Introduction

Constant tissue renewal of stratified squamous epithelia such as skin and oral mucosa is maintained by a balance between cell differentiation and desquamation at the epithelial surface and the replenishment of keratinocytes by cell division occurred in basal and parabasal cell layers[1]. Thus, mitotic activity of keratinocytes is frequently recognizable *in vivo*. In contrast to keratinocytes, fibroblasts in normal adult skin and oral mucosa infrequently divide and are static in nature[2,3]. In addition, in an uninjured tissue, they are relatively inactive in terms of protein synthesis and thin in histological appearance[4]. However, the wound healing process induces phenotypic alterations in the resident fibroblast population from quiescence to migration and proliferation, contracting cells and producing extracellular matrix[5]. Since fibroblasts in a monoculture are invariably highly proliferative in a serum-containing medium, this fibroblast phenotype seems to be "activated" fibroblasts in vivo in response to wound healing[4]. While studies have used proliferating fibroblasts in a monoculture to examine the cytotoxic effects of pharmaceuticals and chemicals and consequent cellular responses[6,7], data obtained from those experiments cannot be extrapolated to the effects in the target tissue in vivo because their cellular responses may be different from those of inactive fibroblasts, similar to the phenotype in an uninjured tissue.

Recently, researchers have paid an attention to tissue engineering as an emerging technology for regenerative medicine. In contrast to a monolayer culture system, it is well-known that cells cultured in a three-dimensional (3D) scaffold behave differently[8,9]. In fact, the proliferation rate and collagen production by fibroblasts embedded in a 3D collagen matrix decreased[10,11]. Those fibroblast phenotypic changes were confirmed to be beneficial to dermal tissue regeneration[12]. Although most products commercially available such as Dermagraft® and Apligraf® incorporate normal proliferating early passaged fibroblasts from foreskin to fabricate the tissue-engineered substitutes, there is a body of data suggesting that use of static fibroblasts is a better model to address biological phenomena than primary proliferating fibroblasts even when fabricating 3D tissue-engineered constructs[5]. Thus, to implement a better model, we should use different culture technique that allows to provide quiescent fibroblasts even in a monolayer culture system.

There have been two means to modulate fibroblast phenotype to a static and non-proliferating nature mimicking residential fibroblasts in dermal tissue. One is utilization of irradiated dermal fibroblasts[13], and the other is use of keratinocyte-conditioned medium (CM)[14]. Previous reports stated skin keratinocyte-CM reduced skin fibroblast proliferation as well as collagen matrix synthesis[2,15,16]. In the oral mucosa, interactions between keratinocyte and fibroblast are also critical to basic research as a tool to translate into regenerative medicine. However, there have been few studies on their cross-talk by using oral keratinocytes-conditioned medium (OK-CM)[17]. Consequently, we are interested in the feasibility of OK-CM that is always discarded in our laboratory.

In this study, we hypothesized the OK-CM yielded in our laboratory might contribute to oral fibroblast (OF) phenotypic changes from an actively-cycling state to a more static state. Thus, this study aimed to examine if the OK-CM can decrease OFs proliferation in a monolayer culture. In addition, we further characterized the phenotypic changes of OFs cultured in OK-CM. The re-use of OK-CM could provide an easier and inexpensive approach for studying the cellular biology of OFs.

Materials and Methods

Procurement of oral mucosa samples

The protocol for obtaining human oral mucosa samples was approved by the Niigata University Faculty of Dentistry Internal Review Board. Patients that had been subjected to third molar removal were given sufficient information regarding this study, and all individuals signed an informed consent form.

Primary oral keratinocyte and fibroblast cultures

At the Oral and Maxillofacial Surgery outpatient clinic at the Niigata University Medical & Dental Hospital, an oral mucosa sample was harvested from the area in adjacent to the site of tooth extraction without causing any morbidity. It was transported in a 15-mL conical tube containing 5 mL of a basic keratinocyte culture medium, EpiLife® (Life Technologies, Carlsbad, CA). For primary culture of oral keratinocyte (OK) and oral fibroblasts (OF), we used our previously reported system[18,19] . Briefly, a tissue specimen was transferred into a 0.04% trypsin solution (Life Technologies, Carlsbad, CA) containing 1.5% of Antibiotic-Antimycotic (Life Technologies) and soaked overnight at room temperature. OKs were mechanically dissociated from the underlying connective tissue in a 0.0125% defined trypsin-inhibitor (DTI)(Life Technologies), resuspended in "complete" EpiLife® (Life Technologies) supplemented with EpiLife Defined Growth Supplements (EDGS) (Life Technologies), 0.06 mM Ca^{++}, Gentamicin (5.0 μg/mL), and Amphotericin B (0.375 μg/mL) (Life Technologies), a chemically defined culture medium, and plated at a density of 4.0-5.0 $\times 10^4$ cells/cm^2. When they reached a 70-80% confluence, they were detached with a 0.025% trypsin/ethylenediaminetetraacetic acid (EDTA) solution, neutralized with DTI, centrifuged and re-plated at a density of 0.7-1.0 $\times 10^4$ cells/cm^2. For primary OF culture, we used an explant culture technique. Small explants were placed in a 60-mm petri dish (Corning, New York, NY), and incubated in a moist atmosphere of 5% CO_2 at 37 degree in a DMEM culture medium (Wako Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Nichirei, Tokyo, Japan), Gentamicin and Amphotericin B (hereafter called as DMEM-CS). Cells were fed every other day. Once the cell outgrowth was sufficient, cells were detached with a 0.025% trypsin/EDTA solution (Life Technologies), neutralized with DTI, then re-plated in another culture vessels (0.8 $\times 10^4$ cells/cm^2). The 3rd to 5th passaged fibroblasts were used in this study.

Preparation of oral keratinocyte conditioned medium (OK-CM).

Conditioned media were generated from near-confluent cultures (80-90 percent) of 2nd or 3rd passaged OKs. The completed Epilife media were conditioned for 24 hours, passed through a 0.45um filter and stored at -80℃ until use.

Proliferation assay

OFs (5×10^3) were plated into 96 microplate-wells with a 100 μL of DMEM-CS. Twenty-four hours later, culture media were changed to DMEM-CS, serum-free DMEM (SF-DMEM) and OK-CM, and OFs were cultured up to 96 hours. To determine proliferating rate of OFs, MTT colorimetric assays (Roche Diagnostics, Indianapolis, IN) were performed at 48, 72 and 96 hours incubation according to the manufacturer's instructions. Optical density was measured at 570 nm with a reference wavelength of 690 nm using a Multi scan FC 96-well plate reader (Thermo Fisher Scientific, Waltham, MA). All assays were performed in triplicate.

Fluorescence-activating cell sorter (FACS) analysis

After OFs grown in a 60mm tissue culture dish were cultured in three media for 48 hours, cells harvested with a 0.025% trypsin/EDTA solution were fixed in 70% ice-cold ethanol. Cell cycle analysis was done as previously described using FACS Aria II (Beckton Dickinson, Franklin Lakes, NJ)[18] . ModFit software (Beckton Dickinson) was used to determine cell cycle distribution.

Senescence-Associated β-Galactosidase (SA-β-gal)

After OFs were cultured in three media for 72 hours, SA-β-gal activity was detected using the SA-β-gal staining kit (Cell Signaling Technology, Inc., Beverly, MA) according to the manufacturer's instructions. After staining, stained and unstained cells were photographed.

Enzyme-Linked Immunosorbent Assay (ELISA)

For characterization of OFs cultured in different media, the selected protein levels secreted by OFs were quantified by ELISA. Supernatants (DMEM-CS, SF-DMEM and OK-CM) were collected on 48 and 96 hours later after media change. Keratinocyte growth factor (KGF) was measured using a Quantikine ELISA kit (R&D systems, Minneapolis, MN). To determine the ability of OFs to synthesize type I collagen, human type I collagen ELISA kit (ACEL, Sagamihara, Japan) was used according to the manufacturer's instructions. Sensolyte® Matrix Metalloproteinase-1 (MMP-1) ELISA kit was purchased from ANASPEC (Fremont, CA) and the levels of MMP-1 in three different media were quantified in accordance with the manufacturer's specifications. All assays were performed in duplicate.

Statistical analysis

Data are all presented as the mean ± standard deviations (SD). The statistical differences among OFs cultured in three different media were determined using a repeated one-way analysis of variance (ANOVA) test. Multiple comparison of the inter-subgroup was adjusted using the Tukey Kramer *post hoc* test. A difference of $p<0.05$ was considered significant.

Results

OK-CM reduced proliferating rate of OFs

The proliferating rate of OFs cultured in OK-CM for 48, 72 and 96 hours was significantly different among three different culture media (Fig. 1). Nevertheless, OFs cultured in OK-CM were still able to proliferate as the optical density of MTT assay increased over time. Likewise, OFs grown in unconditioned (fresh) EpiLife culture medium had a similar proliferating rate compared with OFs in OK-CM (data not shown). The proliferating rates of OFs cultured in SF-DMEM for 72 and 96 hours were lower than that for 48 hours, indicating OFs hardly proliferated in SF-DMEM (Fig. 1).

OK-CM induces slow cell-cycling

Cell cycle analysis revealed that OFs cultured in DMEM-CS were significantly actively-cycling, compared with OFs grown in OK-CM for 48 hours. In other words, the cell population of OFs cultured in OK-CM was slow-cycling, -- quiescent --, because the cell cycle progression was blocked in G0/G1. This was confirmed that fewer cells exited out of G0/G1 phase (84.06% versus 74.21%: Fig. 2), and consequently fewer

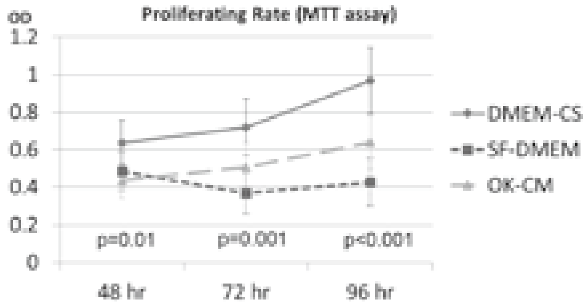

Fig.1 Effects of three different culture media (DMEM-CS, SF-DMEM, OK-CM) on the proliferating rate of human oral mucosa fibroblasts.
The proliferating rate was assessed using a MTT assay (6 samples). The assays were performed in triplicate. There were significant differences of the proliferating rate among three different culture media determined by a repeated one-way ANOVA.

cells progressed to S phase (4.7% versus 13.82%: Fig. 2). The OFs cultured in SF-DMEM also showed a similar profile to the OFs in OK-CM, in contrast to OFs in DMEM-CS. The proportion of cells in G2/M phase was not significantly different among cells in three culture media (Fig 2). There was no indication of an apoptotic cell population because there was no evidence showing any increase in the sub-G1 population of cells (Figure not shown).

Phenotypic change of OFs determined by SA-β-gal activity

To determine if the difference of cell cycle profile among OF populations was due to a senescence-like phenotypic change, we examined the specific expression of SA-β-gal activity. A few OFs cultured in DMEM-CS demonstrated β-gal activity, which was consistent with the higher proliferating rate (Fig. 3A). In contrast, the majority of OFs were stained with β-gal cultured in SF-DMEM (Fig. 3B), indicating replicative senescence. Similar to OFs cultured in DMEM-CS, OFs grown in OK-CM occasionally expressed β-gal while the density of cells grown in OK-CM was lower than OFs monoculture in DMEM-CS (Fig. 3C), indicating the OF cell population in OK-CM is neither post-mitotic nor replicative-senescent but a quiescent phenotype. In addition, the morphology of OFs cultured in OK-CM as well as SF-DMEM became elongated, with thinner cytoplasm, different from spindle-shaped OFs in DMEM-CS. Thus, distinct phenotypic changes of OFs resulted from three

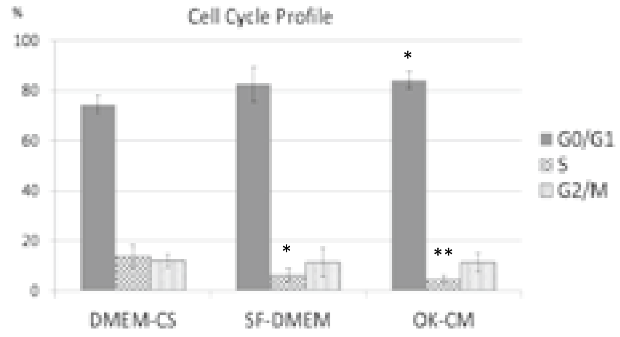

Fig.2 Distribution of human oral fibroblast population in various phases of the cell cycle.
The bar chart shows distributions of cells (%) in G0/G1, S, and G2/M phases as determined by ModFit software. Data are mean percent ± standard deviations (SD) of four independent experiments. The asterisks represent statistically significant differences determined by Tukey's post-hoc test, compared with the profile of cells cultured in DMEM-CS (*$p < 0.05$, **$p < 0.01$).

different culture media.

Comparison of KGF, type I collagen and MMP-1 levels produced by OFs in three different media (Table)

KGF was released by OFs cultured in DMEM-CS and OK-CM whereas the ability of OFs grown in SF-DMEM to secrete KGF was very limited. In addition, KGF produced by OFs in DMEM-CS and OK-CM dramatically increased and decreased over time. OFs cultured in DMEM-CS steadily produced type I collagen over time. In contrast, the ability to synthesize type I collagen by OFs grown in OK-CM and SF-DMEM was significantly lower than OFs in DMEM-CS cultured for up to 96 hours. Furthermore, both OFs cultured in DMEM-CS and OK-CM were able to secret MMP-1 steadily overtime although the amount of MMP-1 produced by OFs grown in SF-DMEM was significantly lower. As a consequence, OFs cultured in DMEM-CS showed a specific pattern of selected protein syntheses.

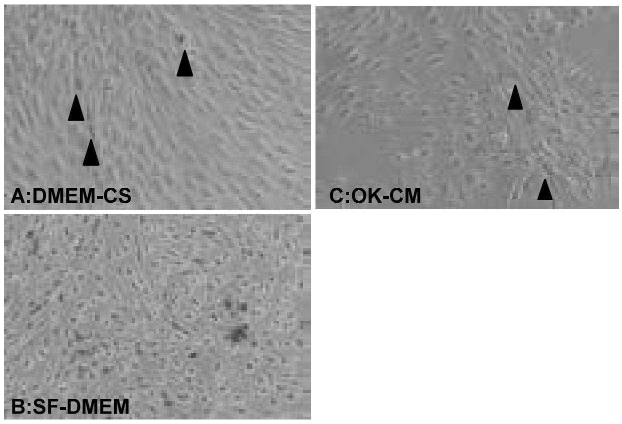

Fig.3 Inverted microscopic images of the SA- β -gal activity of oral fibroblasts grown in three different culture media (DMEM-CS (A), SF-DMEM (B) and OK-CM (C)). In contrast to the spindle-shaped morphology of OFs in DMEM-CS (A), OFs cultured in OK-CM (C) became elongated and thinner. The morphological change was more remarkable in OFs in SF-DMEM (B). Arrowheads in A and C show SA- β -gal positive cells.

		DMEM-CS	SF-DMEM	OK-CM
KGF (pg/mL)	48 hrs	24.7±5.7	0.0±0.0	46.7±35.8
	96 hrs	101.0±26.8	2.3±2.9	24.7±18.1
Type I Collagen (μg/mL)	48 hrs	1.21±0.13	0.35±0.24	0.13±0.14
	96 hrs	0.91±0.38	0.29±0.17	0.33±0.19
MMP1 (pg/mL)	48 hrs	83.7±46.6	2.2±1.1	107.4±36.6
	96 hrs	84.2±59.7	14.4±23.1	103.8±55.0

Table. Levels of KGF, type I collagen and MMP-1 secreted by OFs cultured in three different culture media (DMEM-CS, SF-DMEM, OK-CM) over 96 hours culture period. Data are all presented as the mean ± SD of duplicate measurements in four independent experiments. The asterisks represent statistically significant differences determined by Tukey's post-hoc test (*$p < 0.05$, **$p < 0.01$).

Discussion

This study showed the OK-CM significantly down-regulated the cell proliferation rate of OFs, consistent with the previous study showing the inhibitory effect of skin-keratinocyte CM obtained from a confluent monolayer on skin fibroblast proliferation[2]. Tissue homeostasis depends on essential communications between epithelial cells, stromal cells and the extracellular matrix[20]. With minimal direct cell-to-cell contact, keratinocyte-fibroblast interaction (cross-talk) is controlled mainly by cell-derived soluble factors acting in an autocrine/paracrine manner[16]. In fact, previous investigations showed the regulatory role of soluble factors within skin-keratinocyte CM[21,22]. Harrison et al. speculated skin keratinocytes were capable of secreting growth inhibiting factors for skin fibroblasts[2]. While it is poorly elucidated which cytokine contributes to the suppression of fibroblast proliferation most efficiently, it appears that OKs secret cytokine to suppress OF proliferation. In addition, oral mucosa tissue specific soluble factors may regulate the specific phenotype of OFs because fibroblasts reside in different anatomical regions are known to manifest different phenotypes[5).

Flow cytometric analysis demonstrated that the OFs cultured in OK-CM as well as in SF-DMEM were slower-cycling compared with cells in DMEM-CS, and the decrease in their proliferation rate was associated with inhibition of cell cycle progression at the G1 phase towards S phase transition, resulting in G0/G1 arrest. Since we did not mix any unconditioned, complete EpiLife medium with OK-CM, the deletion of nutrition such as glucose, inorganic ingredients such as calcium and growth factors in the OK-CM might induce the growth arrest in G0/G1 phase. In fact, the glucose levels in the OK-CM were lower than one-third of the unconditioned, complete EpiLife medium, and the calcium concentration in the OK-CM is 0.06mM compared with 1.2mM in SF-DMEM. However, OFs cultured in the unconditioned medium and OK-CM supplemented with 1.2mM calcium also showed a significant decrease of proliferation rate and a similar cell cycle profile (figures not shown). Thus, the reduction of glucose level as well as calcium concentration in the OK-CM was unlikely to involve in the growth arrest of OFs. Although keratinocytes metabolites such as lactate may affect the cell cycle profile, further studies are necessary to find the cause of G0/G1 arrest.

SA-β-gal activity has been used to identify the specific subtypes of heterogeneous fibroblasts between an actively-mitotic cell population and a post-mitotic, non-proliferating, differentiated cell population[23]. With regards to the replicative potential, this result showed that OFs cultured in DMEM-CS and OK-CM exhibited a relatively actively-mitotic phenotype. However, since the cell cycle profile as well as the proliferation rate demonstrated the majority of OFs in OK-CM was quiescent, it would appear that their cycling slowed down, but most of them still retain the cell proliferation potential. In contrast, OFs in SF-DMEM indicated the senescence-like phenotypes, implying the induction of an irreversible post-mitotic phenotypic change. Overall, it is clear that three different culture media used in this study generated the cell populations of OFs showing different phenotypes.

Apart from the proliferating potential, this study also characterized differential ability for OFs cultured in different media to produce and secret the selected growth factors and extracellular matrix components. There have been numerous findings that keratinocytes stimulate fibroblasts to produce growth factors and extracellular matrix including basement membrane components[24]. Post-mitotic fibroblasts have a higher capacity to produce KGF[5] However, this was not in line with the result of the present study because OFs grown in SF-DMEM produced scarce KGF. Keratinocyte-derived interleukin-1 (IL-1) stimulated KGF production by fibroblasts, referred to as a double paracrine pathway[13]. Thus, IL-1 dependent keratinocyte-fibroblast interactions may result in the KGF secretion by OFs cultured in DMEM-CS and OK-CM because of the presence of IL-1 in serum and OK-CM[25,26].

This study also demonstrated the down-regulation of type I collagen synthesis by OFs cultured in OK-CM, consistent with the previous studies[14,15,22]. The collagen synthesis inhibitory effects of OK-CM appeared to be caused by not a single factor but multiple factors such as fibroblast proliferation by mitogens (basic fibroblast growth factor, insulin-like growth factor 1), fibroblast post-mitotic differentiation, several cytokines (IL-1α, β, tumor necrosis factor-α, interferon-α, β, γ) expressed by keratinocytes and anti-fibrogenic factors derived from fibroblasts (transforming growth factor-β1, connective tissue growth factor). Comprehensive analysis of keratinocyte-derived factors is necessary to determine the differences of cytokine profiles between OK-CM and DMEM-CS.

In contrast to the diminution of collagen synthesis by OFs in OK-CM, OK-CM increased MMP-1 expression by keratinocyte-releasable stratifin[27]. The MMP-1 quantification shown in this study was in consistent with the study on stratifin-mediated MMP-1 up-regulation. It would be interesting to assess whether stratifin in the OK-CM induces the similar effect. Although one stated collagen breakdown products may promote new collagen synthesis[28], the OK-CM did not seem to affect new collagen production in this model.

Our primary purpose of this study was to develop of a monolayer fibroblast culture in a more static state for better understandings of OFs biology, and subsequently to examine the feasibility of use of OK-CM to induce their phenotypic changes towards a more quiescent state. However, our ultimate goal is to incorporate this OF population into our clinical protocol in fabricating a tissue-engineered oral mucosa[29] because it is unnecessary to use serum for OF culture and our "chemically-defined" culture system is not disturbed by using OK-CM. We also aim to create *in vitro* models for pathology, toxicology and oral mucosa biology[19] . Different from skin fibroblasts, it was reported that OFs had features of "fetal" wound healing phenotype because of less scar formation in oral mucosa wound[30,31) , suggesting use of slow-cycling OFs provides a more appropriate insight of wound healing by mimicking to an in vivo condition. Thus, further studies are mandatory because the characterization of soluble factors released in OK-CM, an ability to regulate overlying keratinocyte growth/differentiation in an oral mucosa substitute and the observation of phenotypic changes for a longer time period are not elucidated.

In summary, compared with the proliferating population of OFs cultured in DMEM-CS, this study showed significant and specific OK-CM-mediated OF phenotypic changes that include the down-regulation of proliferating rate, the feature of slow-cycling cell population without post-mitotic change, inhibition of type I collagen matrix synthesis and an ability to synthesize KGF and MMP-1. This study suggested the OK-CM generated a quiescent OF population that also possessed the characteristic of extracellular matrix degradation rather than synthesis. Thus, the OK-CM could be feasible for studies of fibroblasts in a 3D environment in vitro.

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